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(57) Abstract

We have discovered a nuclear protein in normal human cells, "retinoblastoma-associated protein 1" ("RBAP-1") that binds directly to the retinoblastoma protein pocket of the underphosphorylated form of the retinoblastoma protein ("RB") and does not bind to phosphorylated RB or to RB with inactivating mutations. The translated RBAP-1 sequence does not resemble other proteins whose sequences are known, and RBAP-1 does not contain a sequence homologous to the transforming element common to viral proteins that bind to the RB pocket. RBAP-1 and the E2F transcription activity have similar DNA-binding specificities and can bind to at least some of the same proteins, such as RB and E4.

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RETINOBLASTOMA-ASSOCIATED PROTEIN 1 cDNA

Background of the Invention

This invention was made in the course of work supported in part by U.S. Government funds, and the government has certain rights in the invention.

This invention relates to tumor suppressor genes.

The human retinoblastoma gene ("*RB-1*") is considered to be the prototype of a class of genes, generally known as "tumor suppressor genes", thought to be involved in suppressing neoplastic growth. Mutations in the retinoblastoma gene and dysfunction of its product have been implicated in the pathogenesis of a wide range of human tumors other than retinoblastomas, including bladder, breast, and small cell lung carcinomas, osteosarcomas, and soft tissue sarcomas. Furthermore, in cell populations where both copies of *RB-1* are mutated, introduction of a wild-type copy of the gene can lead to a decrease in the growth rate or in the tumorigenicity of the cells expressing the exogenous gene (Huang *et al.*, 1988, *Science*, Vol. 242, pp. 1563-1566). The retinoblastoma gene product, "RB", is believed to regulate cell growth, although the manner in which it does so is not well understood.

Several viral transforming proteins, the adenovirus E1A protein ("E1A"), the simian virus large T antigen ("T"), and the human papilloma virus E7 protein ("E7"), bind specifically to RB. The binding of the viral proteins to RB has been mapped to a region of RB termed the "pocket" (Hu *et al.*, 1990, *EMBO J.*, Vol. 9, pp. 1147-1155; Kaelin *et al.*, 1990, *Mol. Cel. Biol.*, Vol. 10, pp 3761-3769; Huang *et al.*, 1990, *EMBO J.*, Vol. 9, pp. 1815-1822). The viral proteins share a short, homologous, colinear, transforming element, having at its core the amino acid sequence LXCXE, that is capable of binding to the RB pocket. A synthetic peptide of this viral element is capable of binding to the RB pocket and when bound blocks the binding of viral proteins to the RB pocket.

Analysis of RB throughout the cell cycle has demonstrated that it is phosphorylated and dephosphorylated at specific stages of the cell cycle. RB is non-phosphorylated, or "underphosphorylated", in the Go and G1 phases and becomes phosphorylated at the start of S phase, the G1/S boundary, and remains phosphorylated throughout S phase, G2 and early mitosis (Buchkovich *et al.*,

1989, *Cell*, Vol. 58, pp. 1097-1105; Chen *et al.*, 1989, *Cell*, Vol. 58, pp. 1193-1198; DeCaprio *et al.*, 1989, *Cell*, Vol. 58, pp. 1085-1095; Mihara *et al.*, 1989, *Science*, Vol. 246, pp. 1300-1303; Xu *et al.*, 1989, *Oncogene*, Vol. 4, pp. 807-812). In terminally differentiated cells and cells that are induced to terminally differentiate, RB is underphosphorylated (Mihara *et al.*, 1989; Furukawa *et al.*, 1990, *Proc. Natl. Acad. Sci. USA*, Vol. 87, pp. 2770-2774). Interaction of the viral transforming proteins with RB is cell-cycle regulated. For example, T does not bind to the phosphorylated form of RB (Ludlow *et al.* 1989, *Cell*, Vol. 56, pp. 57-65), suggesting that some of the growth suppressor functions of RB may be carried out by the underphosphorylated form of RB. The interaction of the viral transforming proteins with RB at specific stages of the cell cycle further supports the proposal that RB is involved in the pathogenesis of some human cancers.

Summary of the Invention

We have discovered a nuclear protein in normal human cells, here termed "retinoblastoma-associated protein 1" ("RBAP-1") that, based on *in vitro* evidence, binds directly to the RB pocket of the underphosphorylated form of RB and does not bind to phosphorylated RB or to RB with inactivating mutations. The direct binding of RBAP-1 to RB suggests that RBAP-1 is involved in the RB signal transduction pathway.

We have fully sequenced a near full length clone of RBAP-1 encoding DNA ("*RBAP-1*"), and deduced the RBAP-1 amino acid sequence. A search of DNA sequence data bases reveals that *RBAP-1* does not resemble other proteins whose DNA sequences are known. The deduced amino acid sequence of RBAP-1 also reveals that RBAP-1 does not contain a colinear sequence, LXCXE, homologous to the transforming element common to viral proteins that bind to RB, although it appears to bind to the same region of RB as do the viral transforming elements.

Analysis of *RBAP-1* gene expression in cell culture demonstrated that *RBAP-1* is expressed primarily during S phase of the cell cycle. RB becomes phosphorylated at the beginning of S phase and we propose that RBAP-1 carries out a function related to the entry into, or traversal of, S phase by the cells.

Analysis of the DNA-binding properties of RBAP-1 has demonstrated that RBAP-1 binds to a DNA sequence that is also bound by cellular extracts that contain an activity known as E2F. E2F activity was originally described as an E1A-targeted component of the functional transcription complex of the adenovirus promoter and was later shown to be normally complexed to cellular proteins in most cell types. E2F has been functionally defined as a transcription factor that is a DNA-binding protein, and more recently has been shown to be a cellular target of RB (Bandara *et al.*, 1991, *Nature*, Vol. 351, pp. 494-497; Chellappan *et al.*, 1991, *Cell*, Vol 65, pp. 1053-1061; Bagchi *et al.*, 1991, *Cell*, Vol. 65, pp. 1063-1072; Chittenden *et al.*, 1991, *Cell*, Vol. 65, pp. 1073-1082). Also, E2F activity containing extracts have been shown to bind to the RB pocket, and this protein complex can be disrupted by E1A or E7. Interestingly, the RB bound E2F activity can recognize more than one DNA sequence (Chittenden *et al.*, 1991). The binding of more than one DNA sequence by the E2F activity suggests that E2F may be a family of proteins.

In one general aspect the invention features a portion of a normal human nuclear protein that is capable of binding to the RB pocket.

In preferred embodiments the human nuclear protein is RBAP-1, having the sequence shown in Fig. 1. In other preferred embodiments RBAP-1 is synthesized *in vitro* using an RBAP-1 encoding DNA, or is made *in vivo* using an RBAP-1 encoding DNA or using the *RBAP-1* gene. In some embodiments the portion of the human nuclear protein is the RB pocket binding portion of RBAP-1, comprising the nucleotide sequence 1191-1397 as shown in Fig. 1, or may be some other portion, and may be the entire RBAP-1 protein.

In another general aspect, the invention features a RBAP-1 encoding DNA. In preferred embodiments the RBAP-1 encoding DNA includes the nucleotide sequence shown in Fig. 1. In some embodiments the invention features a vector containing a portion of the RBAP-1 encoding DNA and may contain the entire RBAP-1 encoding DNA.

In another general aspect the invention features a vector containing a ***RBAP-1*** **gene.**

In another general aspect, the invention features a method for diagnosing a condition of tumorigenicity in a subject, including the steps of obtaining a tissue sample from a subject and detecting the presence of non-wildtype *RBAP-1* encoding gene in the sample, or detecting alterations in the expression of wildtype *RBAP-1* encoding gene in the sample. "Alteration of expression" as used herein includes an absence of expression, or a substantially decreased expression, or an overexpression of the gene.

In another general aspect, the invention features a nucleic acid probe complementary to a portion of a *RBAP-1* gene. The complementary nucleic acid probe, as used herein, can be complementary to any portion of a *RBAP-1* gene including sense and anti-sense strands of the gene, and including coding and non-coding sequences.

In another general aspect, the invention features a ligand capable of binding to the RBAP-1 protein. In preferred embodiments, the ligand can bind to the RBAP-1 protein or to an RBAP-1/RB protein complex. The ligand can be a protein other than RB, a fusion protein, a polypeptide, or a small molecule. "Small molecule", as that term is used herein, means a chemical compound, a peptide, an oligonucleotide, having a sequence other than the sequences known to be bound by the E2F activity, or a natural product. Preferably the small molecule is a therapeutically deliverable substance.

In another general aspect, the invention features a ligand that is capable of altering the activation of a gene by RBAP-1. The ligand may alter gene activation by RBAP-1 by decreasing, the affinity of RBAP-1 for the specific DNA site, or decreasing, RBAP-1 transactivation of the promoter that is downstream from the DNA binding site.

In another general aspect, the invention features a ligand that is capable of disrupting the interaction of a viral transforming protein and RB, while not disrupting the interaction of RBAP-1 and RB.

In another general aspect the invention features methods for assaying for a ligand that is capable of disrupting the interaction of a viral transforming protein and RB, while not disrupting the interaction of RBAP-1 and RB. In one aspect the method comprises the steps of: immobilizing RB on a solid support;

contacting the ligand and a viral transforming protein with the immobilized RB and separately contacting the ligand and RBAP-1, or a RB binding portion of RBAP-1; determining binding of the viral protein to RB and of RBAP-1 to RB in the presence of the ligand.

In another aspect the method comprises the steps of: transforming a first cell with vectors containing a reporter gene having an activatible promoter, and containing DNA encoding RB and RBAP-1 where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain; transforming a second cell with vectors containing a reporter gene having an activatible promoter, and containing DNA encoding RB and one viral transforming protein where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain; culturing the transformed cells in the presence of a ligand and determining the expression of the reporter gene. A preferred "cell" is a cultured eukaryotic cell, such as a yeast, for example *S. cerevisiae*, or a mammalian cell. An "activatible promoter", as used herein, is a promoter having a sequence specific binding site upstream of the transcriptional start site that is activated by the binding of a sequence specific DNA binding domain to the specific site and the proximity of a transactivating domain to the DNA binding domain. Each of these domains is fused to one protein of a pair that can interact to form a protein-protein complex and thus the domains are brought into the proximity required to activate transcription from the gene.

In another aspect the method comprises the steps of: transforming a mammalian cell expressing a viral transforming protein with vectors containing a reporter gene having an activatible promoter, and containing DNA encoding RB and RBAP-1 where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain; culturing the transformed cell in the presence of a ligand and determining expression of the reporter gene.

In another general aspect, the invention features a monoclonal antibody directed to RBAP-1. In preferred embodiments the monoclonal antibody is

directed against a portion of RBAP-1 including the amino acid residues encoded by nucleotides 1191-1655 of RBAP-1 encoding DNA, as shown in Fig 1.

In another general aspect the invention features eukaryotic homologues of RBAP-1. In preferred embodiments that eukaryotic homologues have been cloned using a portion of the RBAP-1 encoding DNA sequence as a probe.

Description of the Preferred Embodiments

Drawings

Fig. 1 is a diagram showing the nearly complete nucleic acid sequence of the RBAP-1 encoding DNA, and the deduced amino acid sequence. A candidate initiator methionine, M, is shown, although the sequence 5′ of the corresponding ATG is open. The underlined sequence is the sequence of RBAP-1 that contains the RB pocket binding site.

Cloning and Characterization of RBAP-1

The following description, presented by way of example, details the cloning and characterization of RBAP-1. It will be appreciated that the genes of proteins that bind to RBAP-1 or the RBAP-1/RB protein complex can be cloned and characterized in an analogous manner.

The RBAP-1 encoding DNA was cloned from a λgt11 expression library using radiolabelled RB ("*RB") as a probe, according to cloning techniques generally known in the art (see for example, Singh *et al.*, 1989, *Biotechniques*, Vol. 7, pp. 252-162). *RB was prepared using the pGEX-2TK plasmid which is a modification of the commercially available expression vector pGEX-2T (Pharmacia). pGEX-2T has been modified to encode a GST fusion protein in which a recognition sequence for the catalytic subunit of cAMP dependent protein kinase from heart muscle was interposed between the GST leader polypeptide and the polypeptide encoded by the inserted cDNA. Briefly, the library was plated at approximately 40,000 pfu/150 mm plate on 30 plates (pfu = plaque forming units). The expression of β-galactosidase fusion proteins was induced using IPTG impregnated nitrocellulose. The nitrocellulose was probed with *RB by incubating the nitrocellulose in a buffered solution containing *RB, and the unbound proteins were washed off. The plaques corresponding to the

fusion proteins bound by *RB were picked and purified using further rounds of hybridization as is standard in the art. The DNA from the pure plaques was prepared and the sequence representing the RB-binding fusion protein was subcloned into pBKS™ (Stratagene) for sequencing. DNA sequencing was performed using a Sequenase™ 2.0 kit (available from United States Biochemical Corp.) according to a protocol provided by the manufacturer.

Analysis of the DNA sequence demonstrated that 4 of the clones contained overlapping DNA sequence and were derived from a common mRNA. Additional clones were obtained by screening another library and rescreening the original library with one of the above clones. The 2465 bp sequence of *RBAP-1* was deduced from examination of multiple clones; the sequence shown in Fig 1 is nearly the full length sequence of *RBAP-1* encoding DNA, but may be missing about 500 bp from the 5′ end, as determined by Northern Blot analysis.

The binding of the fusion proteins that comprise RBAP-1 to RB was characterized *in vitro*. Briefly, the purified λ phage of each fusion protein was plated on a separate plate, and the expression of the β-galactosidase fusion proteins was induced using IPTG impregnated nitrocellulose. The proteins on the nitrocellulose were renatured (see Vinson *et al.*, 1988, *Genes & Dev.*, Vol. 2, pp. 801-806) and probed with *RB by incubating the nitrocellulose in a buffered solution containing *RB. The proteins that were bound by *RB were visualized by autoradiography. The results of the autoradiograph demonstrated that all 4 fusion proteins of RBAP-1 were capable of binding directly to RB.

The ability of these proteins to bind to the RB pocket was determined by probing the nitrocellulose with a radiolabelled non-binding mutant of RB, and with *RB in the presence of a synthetic peptide homologous to the E7, E1A, T, viral transforming element. The results showed that the fusion proteins bound directly to the RB pocket and that the binding of these fusion proteins to RB could be significantly reduced or blocked by the viral transforming element.

Nucleotides 1191-1655 of the RBAP-1 encoding DNA, as shown in Fig. 1, were subcloned into pGEX-2T (see Kaelin *et al.*, 1991), to create a glutathione S-transferase fusion protein ("GST-RBAP-1"), and used to determine whether RBAP-1 could bind to the phosphorylated or the underphosphorylated form of

RB synthesized *in vivo*. Briefly, GST-RBAP-1 was purified from *E. coli* and bound to glutathione-Sepharose™, a glutathione-linked cellulose gel (Pharmacia). RB was prepared from asynchronously growing cells and incubated with the Sepharose™ bound GST-RBAP-1. After washing, bound proteins were eluted from the Sepharose™ and immunoblotted with a monoclonal antibody against RB (monoclonal 245 available from Pharmigen). The results demonstrated that RBAP-1 specifically binds to the underphosphorylated form of RB.

The expression of the *RBAP-1* gene was investigated using Northern analysis. The Northern analysis was performed using a RBAP-1 encoding DNA probe and total RNA obtained from peripheral blood T lymphocytes that were resting (Go cells), blocked at the G1/S boundary, and synchronously growing. The results demonstrated that *RBAP-1* mRNA accumulates when the cells are blocked at the G1/S boundary and falls after S phase.

The physical properties of RBAP-1 were examined using techniques that are well known in the art, and RBAP-1 was determined to have the same DNA-binding sequence specificity as E2F. Briefly, RBAP-1 co-purifies with E2F activity on DNA affinity columns (*see*, Means *et al.*, 1992, *Mol. Cel. Biol.*, Vol. 12, pp. 1054-1063). RBAP-1 immunoprecipitated from cell extracts using the monoclonal antibody against RBAP-1, described herein, and bacterially produced RBAP-1 were shown to contain E2F activity by non-denaturing polyacrylamide gel shift analysis using the E2F DNA-binding site for E2F (*see*, Shirodkar *et al.*, 1992, *Cell*, Vol. 68, pp. 157-166). Additionally, RBAP-1 binds to the adenovirus E4 protein, a protein that is known to specifically bind to E2F, as was demonstrated by the binding of RBAP-1 to a GST-E4 fusion protein using the method described above (Kaelin *et al.*, 1991).

The role of RBAP-1 *in vivo*

Without being limited thereby, we here propose a theory of a role of the RBAP-1 protein *in vivo*. We demonstrated that the *RBAP-1* gene is expressed just prior to the point in the cell cycle that RB is phosphorylated, and that RBAP-1 binds specifically to the pocket of the underphosphorylated form of RB. Moreover, the RB pocket region is frequently mutated in human tumors and the

underphosphorylated form of RB is thought to have tumor suppressing effects in that this form is believed to inhibit the progression of the cell cycle.

We propose two alternative models for the significance of RBAP-1 binding to RB. In one model, if RBAP-1 is present before the onset of RB phosphorylation, the binding of RBAP-1 to RB can lead to RB phosphorylation. In this manner RBAP-1 would act "upstream" of RB in a signal transduction pathway and bring about the phosphorylation of RB. This model is consistent with the observation that loss of function RB mutants are hypophosphorylated *in vivo*, suggesting that cellular ligands of RB must bind to the RB pocket before phosphorylation can occur. Alternatively, RBAP-1 may be a "downstream" target of RB. In this model RBAP-1 binds to dephosphorylated RB generated near the end of M phase or is bound by newly synthesized RB that has not undergone post-translational modification.

Use

The invention provides for identification of ligands that bind to RBAP-1 or the RBAP-1/RB complex, identification of ligands that disrupt the binding of RB to a viral transforming protein, or the viral transforming element, and do not effect the binding of RB to RBAP-1, production of monoclonal antibodies directed to RBAP-1 or any peptide of RBAP-1, and detection of non-wild-type *RBAP-1* genes or detection of alteration in the expression of wild-type *RBAP-1* genes.

Identification of ligands that bind to RBAP-1 or RBAP-1/RB.

The RBAP-1 protein can be used to identify ligands that bind to or interact with RBAP-1 or with the RB/RBAP-1 complex. The identification of ligands that bind to RBAP-1 or the RBAP-1/RB complex can be approached using the same method by which RBAP-1 was cloned. For instance, labelled RBAP-1 or a complex of labelled RBAP-1/RB can be used as probes for expression libraries of fusion proteins, and the DNA encoding the protein that binds to either RBAP-1 or the RBAP-1/RB complex can be cloned generally as described above. Radioactive labelling is a preferred method for convenient labelling of proteins.

RBAP-1 or a complex of RBAP-1/RB can be used to screen a peptide library. The screening of a peptide library can be done using techniques

generally known in the art (see for example Scott *et al.*, 1990, *Science*, Vol. 249, pp. 386-390; Devlin *et al.*, 1990, *Science*, Vol. 249, pp. 404-406; Lam *et al.*, 1992, *Nature*, Vol. 354, pp. 82-84). Briefly, RBAP-1 can be linked to a reporter gene, such as alkaline phosphatase ("AP") by cloning an in-frame fusion of RBAP-1 and AP ("AP/RBAP-1"), and used to screen a library of peptides linked to beads. The binding of AP/RBAP-1 to beads can be determined by staining and the amino acid sequence of the peptide on the bead determined by sequencing with a microsequencer (Lam *et al.*). In another approach, RBAP-1 can be attached to a solid support, such as a petri dish, and an epitope library, a peptide library inserted into a coat protein of filamentous phage such that the peptide is on the surface of the phage capsule, can be passed over the RBAP-1. Successive rounds of binding to RBAP-1 and propagating the phage that bind to RBAP-1 allows the purification of the individual phage clones (Scott *et al.*; Devlin *et al.*). The sequence of the peptide that binds to RBAP-1 can be determined by sequencing the DNA.

An *in vitro* assay for ligands, especially small molecules, that interact with RBAP-1 and alter its binding to DNA can be established, for example, by immobilizing RBAP-1 on a solid support, such as a microtiter tray well. The immobilized RBAP-1 can be incubated with a mixture of a ligand and a labelled DNA fragment, containing a sequence bound by the E2F activity. After incubation, the well can be washed to remove unbound species and the amount of label remaining in the well can be measured. A ligand that binds to RBAP-1 and disrupts the binding of RBAP-1 to the labelled DNA fragment can be detected by an absence of label remaining in the well.

Alternatively, an *in vivo* assay for ligands that bind to RBAP-1 and alter the activation of a gene that is transactivated by RBAP-1 can be established. For example this assay can be accomplished by transforming a cell, such as the yeast cell *S. cerevisiae*, with a reporter gene, such as β-galactosidase, under the control of an activatible promoter that has a sequence bound by the E2F activity upstream of the promoter. The cell is also transformed with a plasmid encoding RBAP-1. These cells can be grown in the presence of the chromogenic substrate X-gal, and the cells will produce a blue pigment if the β-galactosidase reporter

gene is transactivated by RBAP-1 and transcribed. Cells can be cultured in the presence of different ligands and the ability of the ligand to disrupt the transactivation of the reporter gene can be measured by assaying for the disappearance of the blue color from cell colonies. Ligands that alter the activation of a gene by RBAP-1, either by decreasing the binding of RBAP-1 to the DNA or by decreasing the transactivation of the gene by RBAP-1 may be useful for therapeutic treatment of individuals that are lacking functional RB.

Identification of ligands that disrupt RB binding to viral transforming proteins without disrupting RB binding to RBAP-1.

RBAP-1 can be used to identify ligands that bind to RB and disrupt the binding of the viral transforming proteins to RB without affecting the binding of RBAP-1 to RB. The following methods for the identification of ligands are described for purposes of example only, and as will be appreciated methods within the invention may differ in particulars from those described.

An *in vitro* assay for ligands that disrupt the binding of RB and a viral RB binding protein, such as E7, E1A or T, can be established by immobilizing RB, RBAP-1 or a viral RB binding protein on a solid support, such as in a microtiter tray well. For example, RB can be immobilized on the solid support, and a mixture of a ligand and either labelled RBAP-1 ("*RBAP-1") or labelled E7 ("*E7") can be added to the wells of the microtiter plate. After incubation the wells can be washed to remove unbound species and the amount of label remaining in the well determined. A ligand that disrupts binding to RB can be detected by an absence of label remaining in the well. In particular, a ligand that specifically disrupts the binding of RB to E7 would be demonstrated by a lack of label remaining in the well where RB, *E7 and the ligand had been incubated together, and the presence of label in the well where RB, *RBAP-1, and the same ligand had been incubated. Radioactive labelling of the proteins is a preferred method for convenient labelling of proteins.

An *in vivo* assay for ligands can be established, for example, by using the yeast *S. cerevisiae* that contains a reporter gene, such as β-galactosidase, under the control of an activatible promoter, such as a promoter with multiple GAL4 binding sites. *S. cerevisiae* can be transformed with plasmids encoding chimeric

proteins in which the DNA-binding region of GAL4 can be fused to RB ("GAL4-RB") and the transactivating region of VP16 can be fused to E7 ("VP16-E7") (see for example, Fields *et al.*, 1989, *Nature*, Vol. 340, pp. 245-246; Dang *et al.*, 1991, *Mol. Cell. Biol.*, Vol. 11, pp. 954-962). These cells can be grown in the presence of the chromogenic substrate X-gal, and the cells produce a blue pigment if GAL4-RB fusion binds to the VP16-E7 fusion protein. The cells can be cultured in replicate in the presence of different ligands and the ability of the ligand to disrupt the binding of RB and E7 can be evidenced by an absence of blue pigment produced by the cells. In order to confirm a specific interaction between E7 and Rb, the ligand can be tested for its ability to disrupt the binding of RBAP-1 to RB using a VP16-RBAP-1 fusion protein in place of the VP16-E7 fusion protein in a similar assay.

An *in vivo* assay for ligands that disrupt the binding of RB and a viral transforming protein can alternatively be established in a mammalian cell in an analogous manner. For example a cervical carcinoma cell that expresses E7, such as HeLa cells, can be transformed with DNA-binding and transactivating fusion proteins of RB and RBAP-1, and a reporter gene downstream of an activatible promoter. These cells can be grown in the presence of different ligands in order to find a ligand that is capable of restoring the binding between RB and RBAP-1.

Ligands that appear to disrupt the binding of RB to the viral transforming element without disrupting the binding of RB to RBAP-1, can be assayed for the specificity of this disruption by determining their capacity to interfere with the binding of an unrelated pair of binding proteins. If the ligand is unable to disrupt the binding of other binding proteins then it can be concluded that the ligand interacts specifically with either RB or the viral transforming element to disrupt their binding.

A ligand that selectively disrupts RB binding to the viral transforming element may do so by binding to RB in such a way as to prevent the binding of the transforming element without disrupting the normal RB/RBAP-1 interaction, or may bind to the transforming element with a higher affinity than the affinity of RB and the transforming element. In either case such a ligand can be used in

treatment of individuals suffering from a pathologic disease state, such as cervical carcinoma or a malignancy in which the RB signal transduction pathway has been disrupted.

Deletions of the RBAP-1 encoding DNA to define functional portions of the protein.

The RB pocket binding region of RBAP-1 can be further defined by constructing deletions of the RBAP-1 encoding DNA and determining binding of the proteins encoded by these deletion mutants to the RB pocket. Deletion mutations of *RBAP-1* can be constructed from knowledge of the *RBAP-1* sequence using techniques well known in the art. For example, a polymerase chain reaction technique can be used to construct a subclone of a specific portion of the DNA; or a series of deletion constructs, such as 3′ deletions, can be constructed by cutting the DNA at a convenient restriction endonuclease site upstream of the stop codon and digesting the DNA with an exonuclease to produce a series of deletions in the 3′ end of the DNA. Proteins encoded by deletion mutants of *RBAP-1* can be assayed for their ability to specifically bind to the RB pocket as described above.

Monoclonal antibodies against RBAP-1.

Monoclonal antibodies were raised against a peptide of RBAP-1 using techniques generally known in the art (Harlow *et al.*, 1988, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, Chapter 6). As will be understood, a monoclonal antibody against any portion of RBAP-1 can be produced using the techniques described below, or any of the techniques described by Harlow *et al.*

Briefly, the GST-RBAP-1 fusion protein described above, that is composed of nucleotides 1191-1655 of Fig. 1 fused to glutathione S-transferase, was overexpressed in *E. coli* and isolated. The fusion protein was suspended in complete Freund's adjuvant and injected intraperitoneally into mice. Each mouse was boosted with the isolated fusion protein in incomplete Freund's adjuvant by another intraperitoneal injection approximately two weeks later, and serum was collected from the mouse by tail bleed an additional 10 or more days later. The serum was tested for antibodies against RBAP-1, and subsequent rounds of

boosting and bleeding were done as necessary. Serum samples were checked for specific recognition of RBAP-1 by immunoprecipitation of radiolabelled RBAP-1 and the mice which had produced the best response were prepared for hybridoma fusion. The final booster injection was given 3 weeks after the latest boost, and about 3 days prior to hybridoma fusion, the booster was delivered both as an intravenous injection and an intraperitoneal injection. The spleen was removed from the immunized mice and the cells were separated. The spleen cells were fused to myeloma cells with polyethylene glycol, and the fused cells were aliquoted into wells of a microtiter plate. The cells were grown in selective medium to select for the growth of hybridoma cells only. The wells containing colonies of hybridomas were screened by removing a portion of the cell culture supernatant and detecting the secretion of antibodies by antibody capture on permeabilized cells or in solution. The specific hybridoma colony that secretes antibody was cloned by limiting dilution and expanded by growing in successively larger containers.

Detection of non-wild-type *RBAP-1* or alterations in expression of wild-type *RBAP-1*.

The detection of alterations of expression of wild-type *RBAP-1* or the presence of non-wild-type *RBAP-1* in a tissue sample from a subject, using techniques well known in the art, can provide for early diagnosis of a neoplasm. The following methods are presented for purposes of example only, the methods employed can differ from the described methods and remain within the spirit of the invention.

Alterations in the level of *RBAP-1* expression can be detected by a well known technique such as Northern blotting of the *RBAP-1* mRNA.

Mutations in the *RBAP-1* gene, including point mutations and specific deletions or insertions of the coding sequence, the 5′ untranslated region and the 3′ untranslated region, can be detected by cloning and sequencing the *RBAP-1* allele present in the sample taken from the subject. If desired, the *RBAP-1* mRNA can be sequenced directly, or the polymerase chain reaction technique ("PCR") can be used to amplify *RBAP-1* or its mRNA to produce encoding DNA ("cDNA") and the resultant cDNA can be sequenced. PCR can also be used to

selectively amplify a region of the *RBAP-1* allele; this can be especially useful to identify mutations at the splice-donor sites and in the 3′ and 5′ untranslated regions.

Mutations in the *RBAP-1* gene can alternatively be detected using single strand conformation polymorphisms (Orita *et al.*, 1989, *Proc. Natl. Acad. Sci., USA*, Vol. 86, pp. 2766-2770). This technique detects deletions and is sensitive enough to detect nucleotide substitutions. For the analysis, RBAP-1 can be cloned from a sample taken from the subject, or the genomic DNA can be prepared from the sample and either amplified using the polymerase chain reaction technique ("PCR") or directly digested with a restriction endonuclease. If the DNA sample is cloned or prepared by PCR then the sample can be radiolabelled, denatured, and subjected to neutral polyacrylamide gel electrophoresis. The gel can be dried and exposed to film to determine any differences in mobility between the sample from the patient and the wild-type RBAP-1 control sample. If the DNA sample is prepared by digestion of genomic DNA, it is denatured, subjected to neutral polyacrylamide gel electrophoresis, and the single-stranded DNA's are transferred to nitrocellulose or nylon membrane. The transferred DNA's are probed with radiolabelled RBAP-1 and any differences in mobility between the DNA from the sample and the wild-type RBAP-1 control can be visualized by an autoradiographic exposure of the DNA's.

Mutations in the *RBAP-1* gene can also be detected using a nucleic acid probe that is complementary to a portion of *RBAP-1*. This technique is traditionally used to detect point mutations, and one can use a riboprobe (sense or antisense) which is complementary to the wild-type *RBAP-1* gene sequence to detect point mutations in the coding DNA. The riboprobe is first annealed to either mRNA or DNA isolated from the tissue sample, then cleaved with ribonuclease to specifically cleave the riboprobe at mismatches between it and the sample. The cleaved products are separated by gel electrophoresis, and mismatches are detected as segments of the riboprobe smaller than the full length riboprobe. The point mutations can also be detected using a DNA probe. Mutations in the *RBAP-1* gene that have previously been identified can be

detected using allele-specific probes containing a gene sequence corresponding to that mutation. Presence of a specific mutation is confirmed when an allele-specific probe hybridizes with *RBAP-1* sequences from the sample.

Cloning of the genomic RBAP-1 DNA

The RBAP-1 gene can be cloned, for example, by first screening Southern blots of restriction endonuclease digests of genomic DNA from normal peripheral blood lymphocytes with labelled *RBAP-1* encoding DNA to determine the size of the *RBAP-1* gene and determine an appropriate cosmid library with which to pursue the cloning of the gene. The cosmid library can then be screened using conventional techniques with labelled *RBAP-1* encoding DNA and the *RBAP-1* gene can be subcloned into an appropriate plasmid vector, such as pBluescript™ which is a useful cloning vector that contains a polylinker that is flanked by standard primer sequences (Vector), and sequenced.

Cloning of eukaryotic homologues to RBAP-1

The RBAP-1 encoding DNA ("cDNA") can be used to select probes to clone the cDNA or genomic DNA that encodes the RBAP-1 homologue in other eukaryotic species. A "homologue", as that term is used herein, means a protein in another eukaryotic species that has the same functional properties as the RBAP-1 protein in humans. Techniques for cloning homologues to a known gene are generally known in the art. For example, a Southern blot of DNA from a desired eukaryote can be screened at low stringency using a labelled portion of *RBAP-1* probe or a labelled oligodeoxynucleotide, that was chosen based upon the *RBAP-1* sequence, as a probe, and the RBAP-1 homologue can be cloned using an appropriate DNA library from the eukaryote (*see for example*, Sambrook *et al.*, 1989, *Molecular Cloning: A Laboratory Manual, 2nd Ed.*, Cold Spring Harbor Laboratory Press). In another technique the sequence of the *RBAP-1* cDNA can be used to design degenerate oligodeoxynucleotide primers, and a polymerase chain reaction can be conducted using the degenerate primers and DNA from a desired eukaryote (*see for example*, Hanks *et al.*, 1987, *Proc. Natl. Acad. Sci., USA*, Vol. 84, pp. 388-392; Lee *et al.*, 1988, *Science*, Vol. 239, pp. 1288-1291)

Other embodiments are within the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kaelin Jr., William G.
Flemington, Erik
Sellers, William
DeCaprio, James A.
Livingston, David M.

(ii) TITLE OF INVENTION: Retinoblastoma-Associated Protein 1 cDNA

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

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(B) STREET: Exchange Place, 53 State Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: US
(B) FILING DATE: 13-MAY-1992
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Kaplan, Warren
(B) REGISTRATION NUMBER: 34,199
(C) REFERENCE/DOCKET NUMBER: DFCI#236PCT

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 227-5020
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(C) TELEX: 289374

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2456 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGGATCGAGC CCTCGCCGAG GCCTGCCGCC ATGGGCCCGC GCCGCCGCCG CCGCCTGTCA 60

CCCGGGCCGC GCGGGCCGTG AGCGTCATGG CCTTGGCCGG GGCCCCTGCG GGCGGCCCAT 120
GCGCGCCGGC GCTGGAGGCC CTGCTCGGGG CCGGCGCGCT GCGGCTGCTC GACTCCTCGC 180
AGATCGTCAT CATCTCCGCC GCGCAGGACG CCAGCGCCCC GCCGGCTCCC ACCGGCCCCG 240
CGGCGCCCGC CGCCGGCCCC TGCGACCCTG ACCTGCTGCT CTTCGCCACA CCGCAGGCGC 300
CCCGGCCCAC ACCCAGTGCG CCGCGGCCCG CGCTCGGCCG CCCGCCGGTG AAGCGGAGGC 360
TGGACCTGGA AACTGACCAT CAGTACCTGG CCGAGAGCAG TGGGCCAGCT CGGGGCAGAG 420
GCCGCCATCC AGGAAAAGGT GTGAAATCCC CGGGGGAGAA GTCACGCTAT GAGACCTCAC 480
TGAATCTGAC CACCAAGCGC TTCCTGGAGC TGCTGAGCCA CTCGGCTGAC GGTGTCGTCG 540
ACCTGAACTG GGCTGCCGAG GTGCTGAAGG TGCAGAAGCG GCGCATCTAT GACATCACCA 600
ACGTCCTTGA GGGCATCCAG CTCATTGCCA AGAAGTCCAA GAACCACATC CAGTGGCTGG 660
GCAGCCACAC CACAGTGGGC GTCGGCGGAC GGCTTGAGGG GTTGACCCAG GACCTCCGAC 720
AGCTGCAGGA GAGCGAGCAG CAGCTGGACC ACCTGATGAA TATCTGTACT ACGCAGCTGC 780
GCCTGCTCTC CGAGGACACT GACAGCCAGC GCCTGGCCTA CGTGACGTGT CAGGACCTTC 840
GTAGCATTGC AGACCCTGCA GAGCAGATGG TTATGGTGAT CAAAGCCCCT CCTGAGACCC 900
AGCTCCAAGC CGTGGACTCT TCGGAGAACT TTCAGATCTC CCTTAAGAGC AAACAAGGCC 960
CGATCGATGT TTTCCTGTGC CCTGAGGAGA CCGTAGGTGG GATCAGCCCT GGGAAGACCC 1020
CATCCCAGGA GGTCACTTCT GAGGAGGAGA ACAGGGCCAC TGACTCTGCC ACCATAGTGT 1080
CACCACCACC ATCATCTCCC CCCTCATCCC TCACCACAGA TCCCAGCCAG TCTCTACTCA 1140
GCCTGGAGCA AGAACCGCTG TTGTCCCGGA TGGGCAGCCT GCGGGCTCCC GTGGACGAGG 1200
ACCGCCTGTC CCCGCTGGTG GCGGCCGACT CGCTCCTGGA GCATGTGCGG GAGGACTTCT 1260
CCGGCCTCCT CCCTGAGGAG TTCATCAGCC TTTCCCCACC CCACGAGGCC CTCGACTACC 1320
ACTTCGGCCT CGAGGAGGGC GAGGGCATCA GAGACCTCTT CGACTGTGAC TTTGGGGACC 1380
TCACCCCCCT GGATTTCTGA CAGGGCTTGG AGGGACCAGG GTTTCCAGAG ATGCTCACCT 1440
TGTCTCTGCA GCCCTGGAGC CCCCTGTCCC TGGCCGTCCT CCCAGCCTGT TTGGAAACAT 1500
TTAATTTATA CCCCTCTCCT CTGTCTCCAG AAGCTTCTAG CTCTGGGGTC TGGCTACCGC 1560
TAGGAGGCTG AGCAAGCCAG GAAGGGAAGG AGTCTGTGTG GTGTGTATGT GCATGCAGCC 1620
TACACCCACA CGTGTGTACC GGGGGTGAAT GTGTGTGAGC ATGTGTGTGT GCATGTACCG 1680
GGGAATGAAG GTGAACATAC ACCTCTGTGT GTGCACTGCA GACACGCCCC AGTGTGTCCA 1740
CATGTGTGTG CATGAGTCCA TGTGTGCGCG TGGGGGGGCT CTAACTGCAC TTTCGGCCCT 1800
TTTGCTCTGG GGGTCCACAA GGCCCAGGGC AGTGCCTGCT CCCAGAATCT GGTGCTCTGA 1860
CCAGGCCAGG TGGGGAGGCT TTGGCTGGCT GGGCGTGTAG GACGGTGAGA GCACTTCTGT 1920
CTTAAAGGTT TTTTCTGATT GAAGCTTTAA TGGAGCGTTA TTTATTTATC GAGGCCTCTT 1980
TGGTGAGCCT GGGGAATCAG CAAAGGGGAG GAGGGGTGTG GGGTTGATAC CCCAACTCCC 2040
TCTACCCTTG AGCAAGGGCA GGGGTCCCTG AGCTGTTCTT CTGCCCCATA CTGAAGGAAC 2100
TGAGGCCTGG GTGATTTATT TATTGGGAAA GTGAGGGAGG GAGACAGACT GACTGACAGC 2160
CATGGGTGGT CAGATGGTGG GGTGGGCCCT CTCCAGGGGG CCAGTTCAGG GCCCCAGCTG 2220
CCCCCCAGGA TGGATATGAG ATGGGAGAGG TGAGTGGGGG ACCTTCACTG ATGTGGGCAG 2280
GAGGGGTGGT GAAGGCCTCC CCCAGCCCAG ACCCTGTGGT CCCTCCTGCA GTGTCTGAAG 2340
CGCCTGCCTC CCCACTGCTC TGCCCCACCC TCCAATCTGC ACTTTGATTT GCTTCCTAAC 2400
AGCTCTGTTC CCTCCTGCTT TGGTTTTAAT AAATATTTTG ATGACGTTAA AAAAAA 2456

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 437 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ala Leu Ala Gly Ala Pro Ala Gly Gly Pro Cys Ala Pro Ala Leu
1               5                  10                  15
Glu Ala Leu Leu Gly Ala Gly Ala Leu Arg Leu Leu Asp Ser Ser Gln
            20                  25                  30
Ile Val Ile Ile Ser Ala Ala Gln Asp Ala Ser Ala Pro Pro Ala Pro
        35                  40                  45
Thr Gly Pro Ala Ala Pro Ala Ala Gly Pro Cys Asp Pro Asp Leu Leu
    50                  55                  60
Leu Phe Ala Thr Pro Gln Ala Pro Arg Pro Thr Pro Ser Ala Pro Arg
65                  70                  75                  80
Pro Ala Leu Gly Arg Pro Pro Val Lys Arg Arg Leu Asp Leu Glu Thr
                85                  90                  95
Asp His Gln Tyr Leu Ala Glu Ser Ser Gly Pro Ala Arg Gly Arg Gly
            100                 105                 110
Arg His Pro Gly Lys Gly Val Lys Ser Pro Gly Glu Lys Ser Arg Tyr
        115                 120                 125
Glu Thr Ser Leu Asn Leu Thr Thr Lys Arg Phe Leu Glu Leu Leu Ser
    130                 135                 140
His Ser Ala Gly Asp Val Val Asp Leu Asn Trp Ala Ala Glu Val Leu
145                 150                 155                 160
Lys Val Gln Lys Arg Arg Ile Tyr Asp Ile Thr Asn Val Leu Glu Gly
                165                 170                 175
Ile Gln Leu Ile Ala Lys Lys Ser Lys Asn His Ile Gln Trp Leu Gly
            180                 185                 190
Ser His Thr Thr Val Gly Val Gly Gly Arg Leu Glu Gly Leu Thr Gln
        195                 200                 205
Asp Leu Arg Gln Leu Gln Glu Ser Glu Gln Gln Leu Asp His Leu Met
    210                 215                 220
Asn Ile Cys Thr Thr Gln Leu Arg Leu Leu Ser Glu Asp Thr Asp Ser
225                 230                 235                 240
Gln Arg Leu Ala Tyr Val Thr Cys Gln Asp Leu Arg Ser Ile Ala Asp
                245                 250                 255
Pro Ala Glu Gln Met Val Met Val Ile Lys Ala Pro Pro Glu Thr Gln
            260                 265                 270
Leu Gln Ala Val Asp Ser Ser Glu Asn Phe Gln Ile Ser Leu Lys Ser
        275                 280                 285
Lys Gln Gly Pro Ile Asp Val Phe Leu Cys Pro Glu Glu Thr Val Gly
    290                 295                 300
Gly Ile Ser Pro Gly Lys Thr Pro Ser Gln Glu Val Thr Ser Glu Glu
305                 310                 315                 320
Glu Asn Arg Ala Thr Asp Ser Ala Thr Ile Val Ser Pro Pro Pro Ser
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                325                 330                 335
Ser Pro Pro Ser Ser Leu Thr Thr Asp Pro Ser Gln Ser Leu Leu Ser
            340                 345                 350
Leu Glu Gln Glu Pro Leu Leu Ser Arg Met Gly Ser Leu Arg Ala Pro
        355                 360                 365
Val Asp Glu Asp Arg Leu Ser Pro Leu Val Ala Ala Asp Ser Leu Leu
    370                 375                 380
Glu His Val Arg Glu Asp Phe Ser Gly Leu Leu Pro Glu Glu Phe Ile
385                 390                 395                 400
Ser Leu Ser Pro Pro His Glu Ala Leu Asp Tyr His Phe Gly Leu Glu
                405                 410                 415
Glu Gly Glu Gly Ile Arg Asp Leu Phe Asp Cys Asp Phe Gly Asp Leu
            420                 425                 430
Thr Pro Leu Asp Phe
        435
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Claims

1. A RB pocket binding portion of RBAP-1.

2. The RB pocket binding portion of RBAP-1 in claim 1, having an amino acid sequence including a portion of the amino acid sequence shown in SEQ. ID NO. 1.

3. The RB pocket binding portion of RBAP-1 of claim 2, wherein the said RB pocket binding portion comprises the amino acids from 369-437 as shown in SEQ. ID NO. 1.

4. A substantially purified human protein RBAP-1 encoded by a nucleotide sequence, a portion of which nucleotide sequence is shown in Seq. ID No. 1.

5. The substantially purified human protein RBAP-1 of claim 4 wherein said protein RBAP-1 is capable of binding to protein RB.

6. The substantially purified human protein RBAP-1 of claim 4 wherein said protein RBAP-1 is capable of binding directly to an RB pocket region of protein RB.

7. The substantially purified human protein RBAP-1 of claim 6 wherein said protein RBAP-1 is further capable of binding to an underphosphorylated form of protein RB.

8. The substantially purified human protein RBAP-1 of claim 7 wherein said protein RBAP-1 is further incapable of binding to a phosphorylated form of protein RB.

9. The substantially purified human protein RBAP-1 of claim 4 wherein said protein RBAP-1 is capable of binding to an adenovirus E4 protein.

10. The substantially purified human protein RBAP-1 of claim 4, capable of binding to a pocket region of RB protein, said protein lacking a pentapeptide amino acid sequence having amino acids leucine, cysteine, and glutamic, acid in a first, third, and fifth position respectively.

11. The substantially purified human protein RBAP-1 of claim 10 wherein said protein RBAP-1 is further capable of binding to an underphosphorylated form of protein RB.

12. The substantially purified human protein RBAP-1 of claim 11 wherein said protein RBAP-1 is further incapable of binding to a phosphorylated form of RB.

13. The substantially purified human protein RBAP-1 of claim 4, comprising a peptide fragment capable of binding to a pocket domain of RB protein, said peptide fragment being encoded by nucleotides 1191-1397 of Seq. ID No. 1.

14. The substantially purified human protein RBAP-1 of claim 13, wherein said peptide fragment is encoded by a portion of the nucleotides 1191-1397 of Seq. ID No. 1.

15. A substantially purified eukaryotic protein that binds to protein RB having amino acid homology to protein RBAP-1, a portion of which amino acid sequence of said RBAP-1 protein is shown in Seq. ID No. 2.

16. The eukaryotic protein having homology to RBAP-1 in claim 15, wherein said protein was cloned using a probe derived from the RBAP-1 nucleotide sequence, a portion of which nucleotide sequence is shown in Seq. ID No. 1.

17. An RBAP-1 encoding DNA having a nucleotide sequence including the sequence shown in SEQ. ID No. 1.

18. A vector containing the RBAP-1 encoding DNA of claim 16.

19. A vector containing a RBAP-1 gene.

20. A nucleic acid probe complementary to a RBAP-1 gene.

21. A method for diagnosing a condition of tumorigenicity in a subject, comprising the steps of obtaining a tissue sample from the subject and detecting the presence of a non wild-type RBAP-1 encoding gene in the sample.

22. A method for diagnosing a condition of tumorigenicity in a subject, comprising the steps of obtaining a tissue sample from the subject and detecting an alteration in the expression of a wild-type RBAP-1 encoding gene in the sample.

23. A ligand capable of binding to the RBAP-1 protein.

24. A ligand capable of binding to the RB/RBAP-1 protein complex.

25. The ligand of claims 23 or 24 wherein said ligand is a protein.

26. The ligand of claims 23 or 24 wherein said ligand is a fusion protein.

27. The ligand of claims 23 or 24 wherein said ligand is a polypeptide.

28. The ligand of claims 23 or 24 wherein said ligand is a small molecule.

29. The ligand of claim 23 wherein said ligand is capable of decreasing RBAP-1 transactivation of a gene.

30. The ligand of claim 23 wherein said ligand is capable of decreasing the binding of RBAP-1 to its specific DNA binding site.

31. A ligand capable of disrupting the binding of RB and a viral transforming protein wherein said ligand does not disrupt the binding of the RBAP-1 protein and RB.

32. A method for assaying for a ligand that is capable of disrupting the interaction of a viral transforming protein and RB, while not disrupting the interaction of RBAP-1 and RB comprising the steps of;

immobilizing RB on a solid support,

contacting RB with one viral transforming protein in the presence of said ligand,

separately contacting RB with RBAP-1 in the presence of said ligand,

determining binding of the viral transforming protein to RB and binding of RBAP-1 to RB in the presence of said ligand.

33. A method for assaying for a ligand that is capable of disrupting the binding of a viral transforming protein to RB, while not disrupting the binding of RBAP-1 to RB comprising the steps of,

transforming a cell with vectors containing a reporter gene having an activatible promoter, and containing DNA encoding RB and RBAP-1 where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain,

transforming a cell with vectors containing a reporter gene having an activatible promoter, and containing DNA encoding RB and one viral transforming protein where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain,

culturing said transformed cells in the presence of said ligand,

determining expression of said reporter gene.

34. A method for assaying for a ligand that can disrupt the binding of a viral transforming protein to RB, while not disrupting the binding of RBAP-1 to RB comprising the steps of,

transforming a mammalian cell expressing a viral transforming protein with vectors containing a reporter gene having an activatible promoter, and containing DNA encoding RB and RBAP-1 where one of the potential binding partners is fused to a transactivating domain and the other is fused to a site specific DNA binding domain,

culturing said transformed cell in the presence of said ligand,

determining expression of said reporter gene.

35. A monoclonal antibody that is directed to the gene product of any portion of said DNA molecule in claim 23.

36. The monoclonal antibody of claim 35 comprising a monoclonal antibody directed against the peptide encoded by nucleotides 1191-1655 of Fig. 1.

```
   1 GGGATCGAGCCCTCGCCGAGGCCTGCCGCCATGGGCCCGCGCCGCCGCCGCCGCCTGTCA
   1 ..........................................................

     CCCGGGCCGCGCGGGCCGTGAGCGTCATGGCCTTGGCCGGGGCCCCTGCGGGCGGCCCAT
     ..........................M A L A G A P A G G P

 121 GCGCGCCGGCGCTGGAGGCCCTGCTCGGGGCCGGCGCGCTGCGGCTGCTCGACTCCTCGC
  12 C A P A L E A L L G A G A L R L L D S S

     AGATCGTCATCATCTCCGCCGCGCAGGACGCCAGCGCCCCGCCGGCTCCCACCGGCCCCG
     Q I V I I S A A Q D A S A P P A P T G P

 241 CGGCGCCCGCCGCCGGCCCCTGCGACCCTGACCTGCTGCTCTTCGCCACACCGCAGGCGC
  52 A A P A A G P C D P D L L L F A T P Q A

     CCCGGCCCACACCCAGTGCGCCGCGGCCCGCGCTCGGCCGCCCGCCGGTGAAGCGGAGGC
     P R P T P S A P R P A L G R P P V K R R

 361 TGGACCTGGAAACTGACCATCAGTACCTGGCCGAGAGCAGTGGGCCAGCTCGGGGCAGAG
  92:L D L E T D H Q Y L A E S S G P A R G R

     GCCGCCATCCAGGAAAAGGTGTGAAATCCCCGGGGGAGAAGTCACGCTATGAGACCTCAC
     G R H P G K G V K S P G E K S R Y E T S

 481 TGAATCTGACCACCAAGCGCTTCCTGGAGCTGCTGAGCCACTCGGCTGACGGTGTCGTCG
 132 L N L T T K R F L E L L S H S A D G V V

     ACCTGAACTGGGCTGCCGAGGTGCTGAAGGTGCAGAAGCGGCGCATCTATGACATCACCA
     D L N W A A E V L K V Q K R R I Y D I T

 601 ACGTCCTTGAGGGCATCCAGCTCATTGCCAAGAAGTCCAAGAACCACATCCAGTGGCTGG
 172 N V L E G I Q L I A K K S K N H I Q W L

     GCAGCCACACCACAGTGGGCGTCGGCGGACGGCTTGAGGGGTTGACCCAGGACCTCCGAC
     G S H T T V G V G G R L E G L T Q D L R

 721 AGCTGCAGGAGAGCGAGCAGCAGCTGGACCACCTGATGAATATCTGTACTACGCAGCTGC
 212 Q L Q E S E Q Q L D H L M N I C T T Q L

     GCCTGCTCTCCGAGGACACTGACAGCCAGCGCCTGGCCTACGTGACGTGTCAGGACCTTC
     R L L S E D T D S Q R L A Y V T C Q D L
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FIG. 1a

```
841 GTAGCATTGCAGACCCTGCAGAGCAGATGGTTATGGTGATCAAAGCCCCTCCTGAGACCC
252 R  S  I  A  D  P  A  E  Q  M  V  M  V  I  K  A  P  P  E  T

    AGCTCCAAGCCGTGGACTCTTCGGAGAACTTTCAGATCTCCCTTAAGAGCAAACAAGGCC
    Q  L  Q  A  V  D  S  S  E  N  F  Q  I  S  L  K  S  K  Q  G

961 CGATCGATGTTTTCCTGTGCCCTGAGGAGACCGTAGGTGGGATCAGCCCTGGGAAGACCC
292 P  I  D  V  F  L  C  P  E  E  T  V  G  G  I  S  P  G  K  T

    CATCCCAGGAGGTCACTTCTGAGGAGGAGAACAGGGCCACTGACTCTGCCACCATAGTGT
    P  S  Q  E  V  T  S  E  E  E  N  R  A  T  D  S  A  T  I  V

1081 CACCACCACCATCATCTCCCCCCTCATCCCTCACCACAGATCCCAGCCAGTCTCTACTCA
332 S  P  P  P  S  S  P  P  S  S  L  T  T  D  P  S  Q  S  L  L

    GCCTGGAGCAAGAACCGCTGTTGTCCCGGATGGGCAGCCTGCGGGCTCCCGTGGACGAGG
    S  L  E  Q  E  P  L  L  S  R  M  G  S  L  R  A  P  V  D  E

1201 ACCGCCTGTCCCCGCTGGTGGCGGCCGACTCGCTCCTGGAGCATGTGCGGGAGGACTTCT
372 D  R  L  S  P  L  V  A  A  D  S  L  L  E  H  V  R  E  D  F

    CCGGCCTCCTCCCTGAGGAGTTCATCAGCCTTTCCCCACCCCACGAGGCCCTCGACTACC
    S  G  L  L  P  E  E  F  I  S  L  S  P  P  H  E  A  L  D  Y

1321 ACTTCGGCCTCGAGGAGGGCGAGGGCATCAGAGACCTCTTCGACTGTGACTTTGGGGACC
412 H  F  G  L  E  E  G  E  G  I  R  D  L  F  D  C  D  F  G  D

    TCACCCCCCTGGATTTCTGACAGGGCTTGGAGGGACCAGGGTTTCCAGAGATGCTCACCT
    L  T  P  L  D  F  @ .....

1441 TGTCTCTGCAGCCCTGGAGCCCCCTGTCCCTGGCCGTCCTCCCAGCCTGTTTGGAAACAT
     .....

     TTAATTTATACCCCTCTCCTCTGTCTCCAGAAGCTTCTAGCTCTGGGGTCTGGCTACCGC
     .....

1561 TAGGAGGCTGAGCAAGCCAGGAAGGGAAGGAGTCTGTGTGGTGTGTATGTGCATGCAGCC
     .....

     TACACCCACACGTGTGTACCGGGGGTGAATGTGTGTGAGCATGTGTGTGTGCATGTACCG
     .....
```

FIG. 1b

1681 GGGAATGAAGGTGAACATACACCTCTGTGTGTGCACTGCAGACACGCCCCAGTGTGTCCA

CATGTGTGTGCATGAGTCCATGTGTGCGCGTGGGGGGGCTCTAACTGCACTTTCGGCCCT

1801 TTTGCTCTGGGGGTCCACAAGGCCCAGGGCAGTGCCTGCTCCCAGAATCTGGTGCTCTGA

CCAGGCCAGGTGGGGAGGCTTTGGCTGGCTGGGCGTGTAGGACGGTGAGAGCACTTCTGT

1921 CTTAAAGGTTTTTTCTGATTGAAGCTTTAATGGAGCGTTATTTATTTATCGAGGCCTCTT

TGGTGAGCCTGGGGAATCAGCAAAGGGGAGGAGGGGTGTGGGGTTGATACCCCAACTCCC

2041 TCTACCCTTGAGCAAGGGCAGGGGTCCCTGAGCTGTTCTTCTGCCCCATACTGAAGGAAC

TGAGGCCTGGGTGATTTATTTATTGGGAAAGTGAGGGAGGGAGACAGACTGACTGACAGC

2161 CATGGGTGGTCAGATGGTGGGGTGGGCCCTCTCCAGGGGGCCAGTTCAGGGCCCCAGCTG

CCCCCCAGGATGGATATGAGATGGGAGAGGTGAGTGGGGGACCTTCACTGATGTGGGCAG

2281 GAGGGGTGGTGAAGGCCTCCCCCAGCCCAGACCCTGTGGTCCCTCCTGCAGTGTCTGAAG

CGCCTGCCTCCCCACTGCTCTGCCCCACCCTCCAATCTGCACTTTGATTTGCTTCCTAAC

2401 AGCTCTGTTCCCTCCTGCTTTGGTTTTAATAAATATTTTGATGACGTTAAAAAAAA

FIG.1c

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/04654

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; C07K13/00; C12N15/62; C12P21/08
C12Q1/68; G01N33/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; C12Q ; G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 90, no. 8, 15 April 1993, WASHINGTON US pages 3525 - 3529 HUBER HE;EDWARDS G;GOODHART PJ;PATRICK DR;HUANG PS;IVEY-HOYLE M;BARNETT SF;OLIFF A;HEIMBROOK DC; 'Transcription factor E2F binds DNA as a heterodimer.' see page 3525, column 2, line 47 - page 3526, column 1, line 14 see page 3527, column 1, line 20 - page 3529, column 2, line 6 --- -/--	1-20, 23-31, 35,36

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
04 OCTOBER 1993	14 -10- 1993
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	S.A. NAUCHE

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	MOLECULAR AND CELLULAR BIOLOGY vol. 12, no. 12, December 1992, WASHINGTON US pages 5620 - 5631 SHAN, B. ET AL.; 'Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F.' see the whole document	1-36
P,X	CELL vol. 70, no. 2, 24 July 1992, CAMBRIDGE, NA US pages 351 - 364 KAELIN WG JR;KREK W;SELLERS WR;DECAPRIO JA;AJCHENBAUM F;FUCHS CS;CHITTENDEN T;LI Y;FARNHAM PJ;BLANAR MA;ET AL; 'Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties.' see the whole document	1-36
P,X	CELL vol. 70, no. 2, 24 July 1992, CAMBRIDGE, NA US pages 337 - 350 HELIN K;LEES JA;VIDAL M;DYSON N;HARLOW E;FATTAEY A; 'A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F.' see the whole document	1-36
X	WO,A,8 906 703 (DRYJA,T ET AL.; US) 27 July 1989	23,25-28
A	See the abstract, claims 1-5	31-34
X	EMBO JOURNAL. vol. 6, no. 7, 1987, EYNSHAM, OXFORD GB pages 2061 - 2068 YEE, A.S. ET AL.; 'Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex' see page 2067, column 2, line 15 - page 2067, column 2, line 39	24-28

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